

ORIGINAL PAPER

ANALYSIS OF PLASMA MEMBRANE INTEGRITY BY USING OF FLUORESCCEIN LABELED ANNEXIN V AND ITS RELATIONSHIP TO MOTILITY AND VIABILITY OF RAM SPERM AFTER CRYOPRESERVATION**Maria IVANOVA-KICHEVA, Alexander DIMITROV, Ivan NIKOLOV, Denitza DASKALOVA, Malcho PETROV***

Institute of Biology and Immunology of Reproduction "Akad. Kiril Bratanov" Bulgarian Academy of Sciences, bul. "Tzarigradsko shouse" 73, Sofia Bulgaria

*Faculty of Veterinary Medicine, University of Forestry - Sofia, Tel: +359 72 23 81, Fax: +359 2 72 00 22, e-mail: kichevama@abv.bg

Manuscript received: April 16, 2005; Reviewed: May 20, 2005; Accepted for publication: May 20, 2005

ABSTRACT

The present study was conducted to detect sperm apoptosis in fresh, capacitated and frozen ram semen and to determine its relationship with other seminal parameters as motility and viability. The cryopreservation process induces the plasma membrane disruptions, attended with increasing of phosphatidylserine molecules expression on the extracellular surface, wherefore some of the live spermatozoa loss their biological potential to fertilize the egg.

KEY WORD: spermatozoa, cryopreservation, phosphatidylserine, Annexin V.

РЕЗЮМЕ

Настоящите изследвания имат за цел да се направин анализ на процеса апоптозата при свежи, капацитирани и криоконсервирани сперматозоиди от коч и да се установи взаимовръзката с промените в други сперматологични параметри, като подвижност и преживяемост. Криоконсервацията индуцира нарушения в плазмената мембрана, изразени с нарастване на експерсията на молекули фосфатидилсерин на външната и повърхност, при което някои живи сперматозоиди загубват биологичния си потенциал за оплождане на яйцеклетката.

ПОДРОБНО РЕЗЮМЕ

Нарушенията в интегритета на плазмената мембрана при сперматозоидите играе важна роля в процеса на оплождане и това е особено важно след стресови процедури като криоконсервацията. В настоящите изследвания са изследвани трансмембранното движение и нарушенията в подредеността на фосфатидилсерина от плазмената мембрана при свежи, еквилибрани и криоконсервирани сперматозоиди, чрез прилагане на Анексин V теста. Резултатите показват, че при свежа и еквилибрана сперма се запазва функционалността на плазмената мембрана в областта на акрозомата, постакрозомата и тялото на сперматозоидите. Има единични сперматозоиди, показващи по-бледа флуоресценция. Два метода на криоконсервация (С трис-среда – протокол 1 и трис – ПВП среда – протокол 2) водят до значително понижаване в мотилитета на сперматозоидите, както и на процента живи сперматозоиди (An-/6CFDA+), докато нараства процента на апоптичните клетки (An+/6CFDA+), в сравнение със свежите и еквилибрани сперматозоиди. Като разлики са статистически достоверни ($p < 0.05$). Процента на тотално живите сперматозоиди - An-/6CFDA+ намалява от 84.25% за свежата сперма до 18.34% (Протокол 1) и 36.65% (Протокол 2), като в същото време процента мъртви сперматозоиди нараства до 55.27% (Протокол 1) и 29.18% (Протокол 2). От изследваните два протокола за криоконсервация, трис средата с ПВП съхранява в по-висока степен жизнеспособността на сперматозоидите. От изследванията се установява, че не всички живи сперматозоиди имат потенциал да оплодят яйцеклетката, защото при някои от тях ендогенният фосфатидилсерин е представен на външната повърхност на плазмената мембрана (Ann Cy3.18+/6 CF+). Този факт може да даде обяснение за ниската оплодителна способност на сперматозоидите от коч след криоконсервация.

INTRODUCTION

The sperm plasma membrane (PM) is a key structures [16], affected the destructive processes during cryopreservation. The PM molecular components are influenced by different ways to the temperature lowering. The lipids are comparatively sensitive to the low temperature influence, while the proteins are more cryoresistant. In this way the glycolipids and the glycoproteins take an essential place. The influence of the low temperatures upon the cells membrane structures is connected with the increasing of the phospholipids and cholesterol quantity [1]. This is a result of biochemical transformation

of the phospholipids. During the temperature falling the degradation of phospholipids to the mono and diglycerides, which predispose for constitution of the complex defect as triglyceride – protein is occurred. This complex disintegrates during thawing at a temperature about 50°C. It is considered that the forming of the mono and triglyceride is a result of lipid peroxidation. Their presence in PM may lead to changes in the plasma membrane plastic properties, permeability and the cell viability [17, 18]. Furthermore, the lipid phase transitions and phase deviation during cryopreservation determine one unstable membrane, which predisposes to different structural defects and increased permeability [6].

A temperature diapason between -4°C and -20°C, where the lipid phases transitions from liquid - crystalloid stages to gel stage are occurred take a special place in many investigations [1, 6, 3]. This process is accompanied with lipids phase distribution and takes a special place in the membrane defects forming. It is considered that lipases have initial capability for hydrolysis of lipids to gel condition [3]. In as a result of this the phospholipase activity increased suddenly in conditions of lipid phase transition and these is the reason for the forming of the membrane defects. In this way in erythrocytes it was established regular difference in the phospholipids loss [5, 8, 12]. The phosphatidylcholine is the most sensitive to the influence of the low temperature, followed from the cholesterol and cardiolipine, while the phosphatidylserine (PS) doesn't change the values almost during cryopreservation. As it is seen the lipids phase transition attended with their aggregation with proteins, lead to the forming of PM structural defects, which in the hyperosmolarity conditions of the environment, determined the changes of the cells form, together with the macrovasiculation of their membranes [5].

The aim of the present study is to investigate the plasma membrane PS behavior from ram spermatozoa, during cryopreservation, by using of Annexin V, as marker for PS expression on the extracellular surface of the PM and the connection with other spermatozoa properties.

MATERIALS AND METHODS

Semen samples were provided from 6 rams and used for experimental analysis. Only ejaculates with good motility >80% and high % of normal morphology >80% were used in the study. The semen samples were frizzed according to two cryoprotective protocols. Following the 1-th protocol, one part of each semen samples was diluted 1:1 with a cryoprotective medium (glucose 2.01, lactose 8.0, tris 0.280 and 20% egg yolk). The second part was diluted with medium, without egg yolk, but containing

polivinilpirolidon 0.8 (PVP) – Protocol 2. After 210-min equilibration at 4°C, the semen samples were diluted with the second parts of the mediums, containing 9% glycerol and were frizzed according to Nagaze - Niwa technology. After thawing at 39°C the semen samples were investigated for sperm motility, survival rate and functional integrity of PM. For this purpose the fresh equilibrated and cryopreserved over two protocols sperm cells were suspend to concentration 1×10^6 cell per ml. With pen marker were drawn 2 circles of approximately 0.5 cm diameter on special polypro poly-L-lysine coated slides (Sigma, St. Louis, MO). 25 µl of the each tested cells suspension was placed in each circle. Then the slides were placed at a room temperature allowing the spermatozoa to be absorbed to the plates. After the incubation and washing with binding buffer the double label staining solution, containing 6CFDA and Annexin CY3.18. was used for coloring the sperm samples. The excess liquid was removed by carefully touching to the slide of the circle [13]. The results were observed by light and fluorescence microscope, by using correct filters and light source depending on the label.

RESULTS AND DISCUSSION

The four groups of semen samples (fresh, equilibrated and cryopreserved over protocol 1 and 2) were characterized for sperm motility, survival rate at 39°C and functional integrity of PM (Figure 1). Depending on the various cryostorage protocols used the spermatozoa motility and survival rate decreased significantly compared to fresh and equilibrated semen. The two methods of cryostorage led to a significant decrease of sperm motility, as well as of percentage of live (An-/6CFDA+) spermatozoa, whereas the percentage of apoptotic (An+/6CFDA-) cells increased, compared to fresh and equilibrated semen. The differences between the percentages of apoptotic

spermatozoa in fresh semen and after thawing were statistically significant ($p < 0.05$ Figure 1). The percentage of total vital spermatozoa An-/6CFDA+ decreased from 84.25% for fresh semen to 18.34% (Prot 1) and 36.65% (Prot 2), at the same time the percent of necrotic spermatozoa increased to 55.27% (Prot 1) and 29.18% (Prot 2). Surprisingly the significantly differences between the percent of apoptotic spermatozoa according to the protocol 1 and Protocol 2 of cryopreservation (medium with PVP, $p < 0.05$) were registered. The increased necrotic and apoptotic spermatozoa and the decreased number of live sperm cells indicate that almost half of the spermatozoa classified as live, by 6CFDA+ reaction, are effected by deterioration on their plasma membranes, after cryopreservation. During the period of equilibration the sperm motility and the functional integrity of PM protected values near to those of spermatozoa of fresh semen.

The stained with Annexin Cy3.18/6CFDA ram spermatozoa showed a variety of labeling patterns (Figure 2, 3). Those spermatozoa that remained motile were uniformly labeled over the whole head and tail (Figure 2 a) correspond to live – pattern classification. Other patterns observed include uniform staining on the head with strong fluorescence over mid piece and stronger fluorescence over the acrosome and strong fluorescence over whole region of mid piece, or interrupted on some places midpiece. When observed the same fields of the slides under the red fluorescence may be seen that some of the live spermatozoa (Figure 2 b), reacted also with Ann3.18 day, where such cells classified as apoptotic. The spermatozoa that had been cryopreserved showed much reduced green fluorescence in all regional domains (Figure 3 A), in comparison to spermatozoa from fresh semen (Figure 3 a). Moreover, the red fluorescence on the same fields is more intensive (Figure 3 b).

One characteristic feature of the plasma membrane of

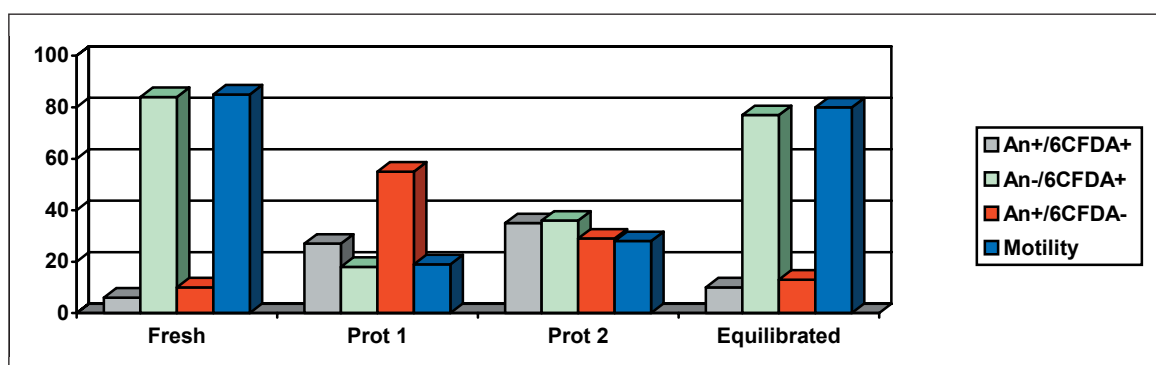


Figure 1. Motility and functional integrity of the plasma membrane in ram spermatozoa before and after cryopreservation.

mammalian sperm cells is the asymmetric transbilayer distribution of lipids that has suggested playing an important role in the fertilization process of spermatozoa [10, 11]. Here, we have investigated the transbilayer movement of PS in the membrane of fresh, equilibrated and cryopreserved ram spermatozoa according to the two cryoprotective protocols to analyze a possible disturbance of the recently characterized transbilayer asymmetry and movement of phosphatidylserine. In order to differentiate population of spermatozoa in the ejaculates, we characterize the expression of PS over the

extracellular surface, as marker of early apoptotic cells [9, 13]. Using this assay in fresh and equilibrated semen about 80% of cells was defined as intact cells, while only 6-12% was apoptotic. Following cryopreservation over two protocols, however the relative amount of apoptotic and necrotic cells significantly decreased compared to fresh semen. Moreover, the half part of live cells seems to be apoptotic and this occasionally explaining the low fertility potential of the thawed ram spermatozoa.

After cooling of the biological membrane, a reordering of membrane components is likely [6]. The cryopreservation

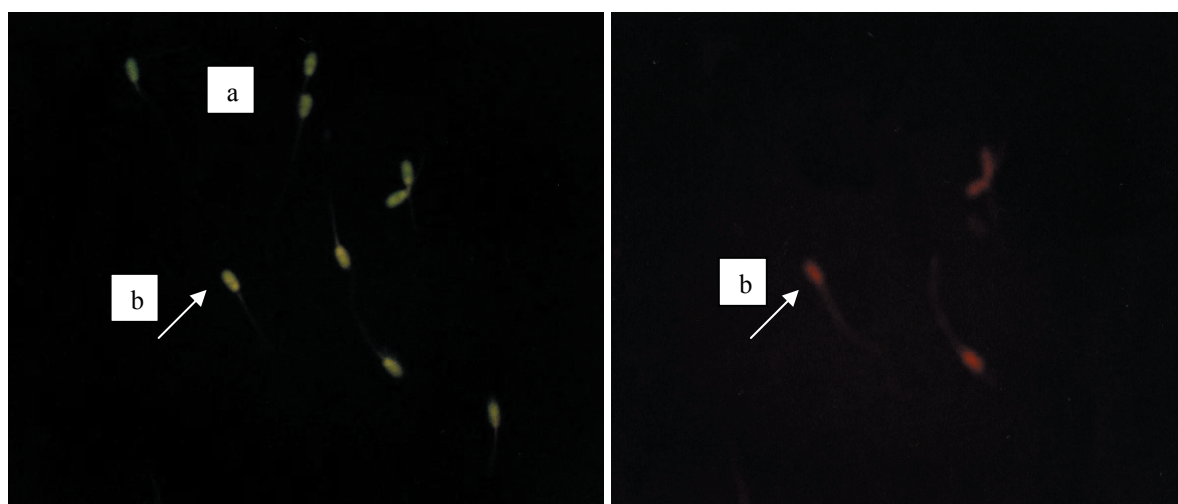


Figure 2. Fresh ram spermatozoa stained by Annexin V test (Ann Cy3.18 - red fluorescence and 6CF - green fluorescence) Different fluorescence patterns observed – a. it is seen strong green fluorescence on head and tail regions typical for live spermatozoa; b – number of spermatozoa, which are stained with the 6CF, were stained at the same time by Ann Cy3.18. These cells are labeled as apoptotic.

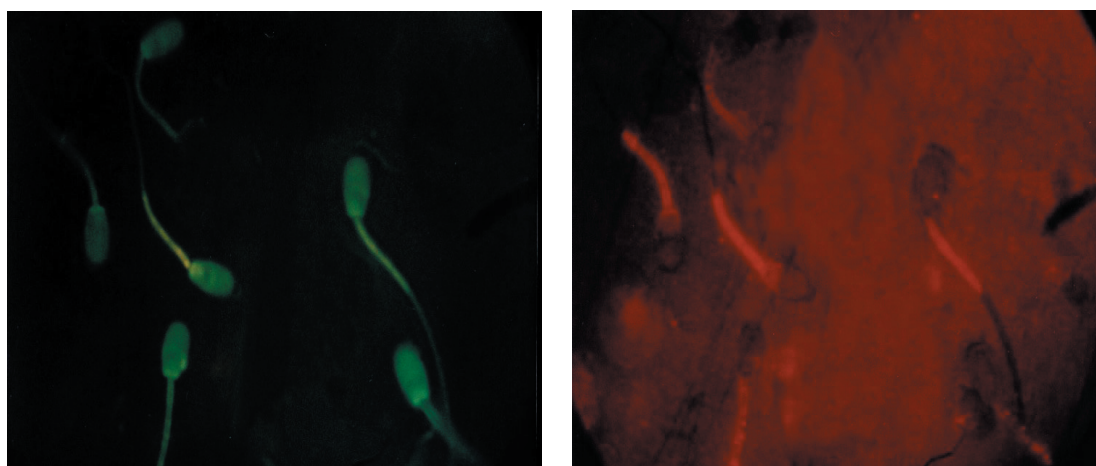


Figure 3. Comparison of green – 6CF (A) and red – Ann Cy3.18 (B) fluorescence in frozen - thawed semen samples. Note that the more of the motile live spermatozoa (green fluorescence - a) were stained by Ann Cy3.18 (strong red fluorescence - b) also.

usually caused sublethal cryodamage to spermatozoa, decreasing post-thaw cell viability [2]. More precise information is needed on the exact proportion of only slightly disturbed spermatozoa and how this ratio might be affected by cryopreservation [7]. Despite different methodological improvements for cryopreservation of ram spermatozoa, the frozen semen has significantly lower fertility than fresh. The present results demonstrate that even when ram spermatozoa survive freezing apparently undamaged, lipid diffusion of their PM is significantly compromised. Such changes, albeit subtle, would have an important iterative effects on the recovery of fertilizing capacity as they would affect phenomena such as spatially depended signaling pathways required for acrosomal exocytosis, development of membrane fusigenicity and antigen migration [14]. It is not clear in present whether the cryoprotectants as egg yolk are having a direct effect or whether low temperature, or both cause the changes. Whatever the reasons, they are not irreversible in spermatozoa cryopreserved in medium with PVP and probably restored the lipid infusibility over the whole membrane to near normal level. PVP has found a range of use as non-penetrating cryoprotectant, for preserving the activity of purified enzymes and for preserving cells sticking none specifically to plastic ware [15]. Its effect in the present context is probably indirect, e.g. such as displacing loosely-bound proteins (most likely seminal plasma proteins) from the surface membrane thereby altering its properties [7].

CONCLUSION

In conclusion the cryopreservation process induces the PM disruptions, attended with increasing of PS expression on the extracellular surface. The Annexin V – binding assay is more sensitive method and gives additional information for alteration or dysfunctional PM, than other supravital staining techniques. Furthermore, immotile spermatozoa were occasionally stained in the annexin- binding assay, suggesting that not all of the immotile spermatozoa represent irreversibly dead cell [4]. On the other hand not all of the live spermatozoa have potential to fertilize the egg, because in some of them the endogenous PS is given to be expressed over the exoplasmic PM leaflet of impaired cells (Ann Cy3.18+/6 CF+). Additionally the Annexin V – binding assay gives possibility to differentiate all cells subpopulations in the ejaculates. It will be interest in future studies to investigate the effects of other macromolecules on lipid diffusion in low temperature stressed sperm membranes.

ACKNOWLEDGEMENTS

Supported in part by grant for research project by Ministry of Education and Science, Bulgaria

REFERENCES

1. Пушкарь НС., Иткин ЮЯ., Низкотемпературная кристаллизация в биологических объектов. Биофизика (1972). 181, 2: 389-391. (Ru)
2. Alvares JG, Storey BT. Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polys as sole cryoprotectant. J Androl (1993). 14, 199-209.
3. Drost - Hansen W. Phase transition in biological systems: manifestations of cooperative processes in vicinal water. Ann NY Acad Csi (1973). 204, N1: 100-108.
4. Glander HJ, Schaller J. Binding of annexin V to plasma membrane of human spermatozoa: a rapid assay for detection on membrane changes after cryostorage. Mol Hum Reprod (1999). 5(2), 109-115.
5. Glaser R. Factor influencing the form of human erythrocytes. Byohpys Membrane Transp (1976). 147-184.
6. Hammersted RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl (1990). 11, 73-88.
7. James PS, Wolfe CA, Mackei A, Ladha S, Prentice A, Jones R. Lipid dynamics in the plasma membrane of fresh and cryopreserved human spermatozoa. Hum Reprod (2002). 14(7), 1815-1827.
8. Kannagi R., Koizumi K. Effects of different physical states of phospholipids substrates on partially purified platelets phospholipase A₂ activity. Biochin Biophys Acta (1979). 556,3: 423-434.
9. Martin S. J., Reutelingsperger, C. P. M., McGahon A. J. et al. Earlier distribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of initial stimulus: inhibition by over expression of Bcl-2 and Abl., J. Exper. Med, (1995). 182, 1545-1556.
10. Muller K, Pomorski T, Muller P, Zachowski A, Herrmann A. Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution of phospholipids in the plasma membrane of ram sperm cells. Biochemistry (1994b). 33: 9968-74.
11. Nolan J, Hammerstedt RH. Regulation of membrane stability and acrozone reaction in mammalian spermatozoa. FASEB J, (1997). 11, 670-682.

12. Strong PN, Kelly RB. Membrane undergoing phase transitions are preferentially hydrolyzed by betabungarotoxin. *Biochem Biophys Acta* (1977). 469, 2 : 231-235.
13. Vermes I., Haanen C. and Reutelingsperger C. P. M. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labeled annexin, 180, (1995). 39-52.
14. Yanagamashi R. Mammalian fertilization. In Knobi E and Neil JD (eds) *The physiology of reproduction*, 2-th ed. Raven Press, New York (1994). 189-317.
15. Watson PF. Artificial insemination and the preservation of semen. In Lamming FE (ed) *Marshall's Physiology of Reproduction* (1990). 2, 747-869.
16. Bevers EM, Comfurius P, Dekkers DWC, Zwaal RFA. Lipid translocation across the plasma membrane of mammalian cells. *Biochim Biophys Acta* (2000). 1439: 317-330.
17. Barosso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Human reproduction* (2000). vol1 5 (6): 1338-1344.
18. Alvarez JG, Storey Bt. Evidence of increased lipid peroxidative damage and loss of superoxide dismutase activity as a model of sublethal cryodamage to human sperm during cryopreservation. *J Androl* (1992). 13: 232-241.